Spectroscopic studies on Tb³⁺ binding to S-100a protein*

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Direct binding assay and fluorescence studies revealed that S-100a protein binds 2 mol of Tb³⁺/mol of protein at pH 6.6. The protein binds Tb³⁺ much more tightly than Ca²⁺, and the upper limit of the observed K_d value for Tb³⁺ is 3.5×10^{-6} M. The Tb³⁺-binding site on the protein must be close to a tyrosine residue, as indicated by fluorescence excitation and emission spectra, where energy transfer from tyrosine is noted. Addition of Tb³⁺ resulted in a conformational change in the protein, as revealed by u.v.-difference spectroscopy and c.d. studies. Far-u.v. c.d. studies indicated the helical content to decrease from approx. 39% to 35% in the presence of Tb³⁺. From u.v.-difference-spectroscopy results the single tryptophan and the tyrosine chromophores in S-100a protein are blue-shifted (i.e. exposed to the solvent) in the presence of Tb³⁺ and the observed conformational changes are similar to those induced by Ca²⁺, suggesting that Tb³⁺ can be employed as a Ca²⁺ analogue in spectral studies with S-100a protein.

INTRODUCTION

In recent years a number of investigators have shown that trivalent lanthanide ions (Ln³⁺) can replace Ca²⁺ at metal-ion-binding sites in many proteins (Brittain *et al.*, 1976; Martin & Richardson, 1979). These ions are useful as reporter groups, since several are either paramagnetic or fluorescent. The ability of Ln³⁺ to replace Ca²⁺ in a specific manner results from similarities in their chemistries. For instance, both Ca²⁺ and Tb³⁺ ions have an ionic radius of approx. 0.1 nm (1 Å) with co-ordination numbers between six and eight, and both exhibit preference for co-ordination with oxygen atoms rather than nitrogen atoms (Shannon & Prewitt, 1969). One of the best spectroscopic Ln³⁺ probes of Ca²⁺-binding sites in proteins is Tb³⁺ luminescence. The luminescence of Tb³⁺ in aqueous solution on a molar basis is quite low, but its fluorescence quantum yield increases dramatically if its binding site on a protein is close to an intrinsic chromophore such as tyrosine, phenylalanine or tryptophan, and this results from radiationless energy transfer (Förster, 1948).

Bovine brain S-100 protein is actually a mixture of two major components, S-100a and S-100b, with subunit compositions of $\alpha\beta$ and β_2 respectively (Isobe & Okuyama, 1981a). Amino acid sequence analysis (Isobe & Okuyama, 1981b) revealed that there is an extensive homology (58%) between the two subunits and that they are evolutionarily related to calmodulin (Cheung, 1980; Kasai *et al.*, 1980), troponin C (Van Eerd & Takahashi, 1975) and parvalbumin (Pechère & Thatcher, 1971). Each of the subunits contains an EF-hand-type Ca²⁺-binding site (Tufty & Kretsinger, 1975) at the C-terminus. S-100 proteins undergo a conformational change upon binding Ca²⁺, and this has been demonstrated by u.v.-difference, c.d. and fluorescence studies as well as hydrodynamic measurements (Mani *et al.*, 1982; Mani & Kay, 1983a,b, 1984; Baudier & Gérard, 1983; Umekawa *et al.*, 1983). In the present investigation for the first time we have studied the Tb³⁺-binding properties of S-100a protein, and the spectroscopic results reveal that the

structural effects of Tb³⁺ binding are very similar to those induced by Ca²⁺ binding. At pH 6.6 the protein binds 2 mol of Tb³⁺/mol of protein (M_r 21 000) with an affinity in the micromolar range.

MATERIALS AND METHODS

Bovine brain S-100a protein was prepared as described previously (Leung *et al.*, 1986). U.v.-absorption and u.v.-difference spectra were recorded on a Perkin–Elmer Lambda 5 spectrophotometer, and c.d. measurements were taken on a Jasco J500C instrument fitted with a DP-500N data processor, as outlined in a previous paper (Leung *et al.*, 1986). Protein concentrations were determined from absorbance measurements by using an $A_{1\%}^{1\text{cm}, 278}$ value of 5.4 (Mani & Kay, 1983b). A Perkin–Elmer model MPF-44B recording spectrofluorimeter was used for fluorescence measurements made at 25 °C. The instrument was operated in the ratio mode with 5 nm bandwidths for excitation and emission slits. The A_{278} of the sample was 0.10 or less. All the solvents used in the optical studies were routinely passed through a Chelex-100 column. The protein was initially dialysed against 0.1 M-Pipes buffer, pH 6.6, containing 2 mM-EDTA at 4 °C, and subsequent dialysis was carried out with at least four changes against the solvent (0.1 M-Pipes buffer, pH 6.6) that had been passed through a Chelex-100 column. The rationale for using Pipes buffer is that it contains only nitrogen donors as opposed to oxygen ligands, to which Tb³⁺ preferentially binds. In addition, it is a very good buffer at pH values slightly below neutrality.

Tb³⁺-binding assays were carried out with the procedure described for the Ca²⁺-binding assay with S-100b protein (Mani & Kay, 1986), with the use of the Centricon 10 microconcentrators from Amicon as ultrafiltration devices to perform rapid flow dialysis as a means of monitoring Tb³⁺ binding to S-100a protein (Barela & Sherry, 1976). S-100a protein was incubated for 15 min with excess Tb³⁺ and was then centrifuged in a Beckman model L centrifuge at 4000 rev./min for 30 min.

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Fig. 1. (a) Fluorescence emission spectrum of Tb^{3+} -S-100a protein in 0.1 M-Pipes buffer, pH 6.6, with excitation at 280 nm, and (b) excitation spectrum of Tb^{3+} -S-100a protein in 0.1 M-Pipes buffer, pH 6.6, with emission at 543 nm

The protein concentration was 0.07 mg/ml.

Free Tb^{3+} in the supernatant (in this case the solution centrifuged across the dialysis membrane) was analysed by using dipicolinic acid (pyridine-2,6-dicarboxylic acid), in accordance with Barela & Sherry (1976). S-100a protein was initially dissolved in 50 mM-Pipes buffer, pH 6.6, containing 2 mM-EDTA and was subjected to exhaustive dialysis against Chelex-100-column-treated 50 mM-Pipes buffer, pH 6.6. Protein (20–30 μmol) was placed in a pre-washed Centricon 10 microconcentrator along with a known amount of Tb^{3+} (3-fold molar excess), the mixture was incubated for 15 min and the solution was centrifuged. The supernatant was assayed for Tb^{3+} by using dipicolinic acid by monitoring the Tb^{3+} -induced fluorescence changes at 545 nm upon excitation at 280 nm on a Perkin-Elmer spectrofluorimeter. A standard curve was generated from the known amount of Tb^{3+} added plotted versus fluorescence intensity at 545 nm. From the amount of free Tb^{3+} found in the supernatant (from the standard curve) along with the total Tb^{3+} added and the amount of S-100a protein used, it was possible to calculate the amount of Tb^{3+} bound to the protein.

RESULTS AND DISCUSSION

S-100a protein, composed of two polypeptide chains (α and β), undergoes a conformational change upon binding Ca^{2+} , and this has been demonstrated by spectroscopic and hydrodynamic measurements (Mani & Kay, 1983a,b, 1984). At neutral pH the protein binds 2 mol of Ca^{2+} /mol as predicted from its amino acid composition. In accord with u.v.-difference spectroscopy, addition of Ca^{2+} to the protein resulted in exposing to the solvent one or more tyrosine residues and the single tryptophan residue located at the C-terminal end of the α -chain.

The Tb^{3+} -binding assay, as described in the Materials and methods section, revealed the binding of 2 mol of Tb^{3+} /mol of S-100a protein at pH 6.6, suggesting that at this pH one Tb^{3+} ion binds per polypeptide chain, since S-100a protein is composed of two polypeptide chains (α

and β), each of which is believed to possess an EF-hand-type Ca^{2+} -binding site. Tb^{3+} binding to S-100a protein is also reflected in fluorescence studies. As shown in Fig. 1, excitation of S-100a protein at 280 nm in the presence of Tb^{3+} exhibited energy transfer from tyrosine, resulting in characteristic Tb^{3+} emission with peaks at 490, 543 and 586 nm. Energy transfer is also indicated by the excitation spectrum of Tb^{3+} -S-100a protein, which shows a large 276 nm tyrosine peak (Fig. 1). Fluorescence titration was carried out by exciting the protein at 280 nm and monitoring the fluorescence intensity at 545 nm as a function of added Tb^{3+} with a view to elucidating the binding stoichiometry. As indicated in Fig. 2, the fluorescence intensity increased linearly with Tb^{3+} addition until the metal ion/protein molar ratio reached 2:1. Subsequent addition (up to a molar ratio of 3:1) did not induce any significant increase in fluorescence intensity at 545 nm, but when the metal ion/protein ratio approached 4:1 the protein was pre-

Fig. 2. Fluorescence titration of S-100a protein with Tb^{3+}

The concentration of S-100a protein was 4 μM in 0.1 M-Pipes buffer, pH 6.6. The excitation wavelength was 280 nm and the emission wavelength 545 nm.

Fig. 3. Percentage change in fluorescence intensity at 545 nm as a function of total Tb³⁺ concentration for S-100a protein in 0.1 M-Pipes buffer, pH 6.6, with excitation at 280 nm

cipitated out. For this reason, even in binding studies, only a 3-fold molar excess of Tb³⁺ was added initially. The observed metal ion/protein binding stoichiometry of 2:1 is in agreement with direct Tb³⁺-binding studies. Fluorescence titration was also carried out for the purpose of determining the affinity with which the protein bound Tb³⁺. The monophasic titration curve obtained in Fig. 3 indicates the existence of one class of Tb³⁺-binding sites at pH 6.6. Since only the total Tb³⁺ concentration is known, an upper limit for K_d was estimated, which is in the micromolar range (K_d $3.5 \times 10^{-6} \pm 0.5 \times 10^{-6}$ M). Nevertheless, the result obtained clearly demonstrates that the protein binds Tb³⁺ very tightly, and this is further supported by the observed linear plot of fluorescence intensity versus metal ion/protein ratio, shown in Fig. 2. This observed upper limit for K_d for Tb³⁺ is higher than that for Ca²⁺ by nearly two orders of magnitude (K_d for Ca²⁺ is 1.2×10^{-4} M at pH 7.5). In accordance with published data in the literature, Ca²⁺-binding proteins in general bind Tb³⁺ with a higher affinity, although both cations have an ionic radius of approx. 0.1 nm, and this conceivably could be due to the tervalency of Tb³⁺ ions, which gives them a much higher charge/volume ratio.

C.d. studies

Typical far-u.v. c.d. spectra of S-100a protein in 50 mM-Pipes buffer, pH 6.6, in the absence and in the presence of Tb³⁺ are shown in Fig. 4. For the apoprotein, the observed $[\theta]_{222}$ value at pH 6.6 was only $-11\,500 \pm 300$ degree·cm²·dmol⁻¹ as opposed to a value of $-16\,000 \pm 300$ degree·cm²·dmol⁻¹ obtained at pH 7.5, implying that protons by themselves can induce an effect similar to Ca²⁺ ions, since Ca²⁺ addition is known to decrease the negative ellipticity at 222 nm. However, the pH effect is significantly higher than the Ca²⁺ effect, since the observed decrease in ellipticity at 222 nm with lowering pH was nearly 25%, whereas Ca²⁺ addition resulted in lowering of the $[\theta]_{222}$ value by only about 10%. (The experimental error involved in measuring the ellipticity value as less than 2%.) The effect of lowering pH on S-100a protein conformation is

Fig. 4. Far-u.v. c.d. spectra of S-100a protein in the absence of Tb³⁺ in 50 mM-Pipes buffer, pH 6.6 (—), and in 50 mM-Pipes buffer, pH 6.6, containing Tb³⁺ (---) at a Tb³⁺/S-100a protein molar ratio of 2:1

The protein concentration was 0.57 mg/ml.

similar to previous observations made with the muscle Ca²⁺-binding protein troponin C in our laboratory (Hincke *et al.*, 1978; McCubbin *et al.*, 1986). However, in the case of troponin C, lowering of pH resulted in an increase in negative ellipticity at 222 nm, and this is because with troponin C the addition of Ca²⁺ also increases the negativity of the $[\theta]_{222}$ value. Thus it is obvious that with both proteins protons can mimic the Ca²⁺ effect. In other words, lowering of pH or the addition of Ca²⁺ had a similar effect on the gross protein conformation. Addition of Tb³⁺ at pH 6.6 caused approximately a 10% decrease in the $[\theta]_{222}$ value to $-10\,500 \pm 300$ degree·cm²·dmol⁻¹, and at this pH the Tb³⁺ effect was greater than that of Ca²⁺, since with Ca²⁺ we noted only a 5% decrease in $[\theta]_{222}$ value. Analysis of the c.d. data by using the method of Chen *et al.* (1974) showed that the α -helical content decreased from 39% to 35% in the presence of Tb³⁺. The error limits in estimating helix content from the observed experimental data were less than 1%, since the error involved in measuring $[\theta]_{222}$ is only 300 degree·cm²·dmol⁻¹.

Fig. 5 reveals the effect of Tb³⁺ on the aromatic c.d. spectrum of S-100a protein at pH 6.6. The negative band around 295 nm is due to tryptophan. The two bands at 284 and 278 nm can be assigned to the tyrosine residues, and the two bands at 268.5 and 261.5 nm can be attributed to the phenylalanine residues. From the spectrum it is obvious that tyrosine residues in the 280 nm region are the ones that are most perturbed by the addition of Tb³⁺.

U.v.-difference spectroscopy

The possible effect of Tb³⁺ on the tryptophan, tyrosine and phenylalanine aromatic groups was investigated by

Fig. 5. Aromatic c.d. spectra of S-100a protein in the absence of Tb^{3+} in 50 mM-Pipes buffer, pH 6.6 (—), and in 50 mM-Pipes buffer, pH 6.6, containing Tb^{3+} (---) at a Tb^{3+} /S-100a molar ratio of 2:1

The protein concentration was 1.29 mg/ml.

Fig. 6. U.v.-difference spectrum of S-100a protein produced by Tb^{3+}

Chelex-treated S-100a protein in 0.1 M-Pipes buffer, pH 6.6, was used, and the Tb^{3+} /S-100a protein molar ratio was 1:1. The protein concentration was 1.33 mg/ml.

measuring u.v.-difference spectra. Fig. 6 shows the difference spectra of S-100a protein when Tb^{3+} was added to the sample cell, compared with non-treated protein in the reference cell. The difference peak at 293 nm is characteristic of a blue-shift of the tryptophan absorption band. The double negative peak at 285 and 278 nm is due to tyrosine absorption bands, and the blue-shifts associated with tryptophan and tyrosine residues are usually interpreted as arising from an increased exposure of these aromatic groups to solvent (Donovan, 1969). It is apparent from Fig. 6 that one or more phenylalanine residues are also perturbed. These findings are very similar to what has been observed with Ca^{2+} with this protein (Mani & Kay, 1983a), except that the magnitude of the observed blue-shift is considerably less, presumably owing to the lower pH of 6.6 used in the present investigation. In addition to lower pH the ratio of Tb^{3+} to S-100a protein used was only 1:1 for these studies, because at a Tb^{3+} /S-100a protein ratio of 2:1 it was not possible to achieve reliable u.v.-absorption-difference spectra. This was due to the higher protein concentration required for these studies, which resulted in the development of turbidity upon Tb^{3+} addition. This, of course, was not a problem with fluorescence studies, since the A_{278} of the protein sample was only around 0.05.

In summary, S-100a protein binds 2 mol of Tb^{3+} /mol of protein at pH 6.6 with an affinity in the micromolar range (K_d 3.5×10^{-6} M), inducing a conformational change affecting its overall secondary structure, as indicated by far-u.v. c.d. studies. The fact that S-100a protein binds Tb^{3+} is very useful, since it can be used in future fluorescence studies to measure the distance between the metal ion- (Ca^{2+} -)binding site and the donor chromophore, which happens to be a tyrosine residue.

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